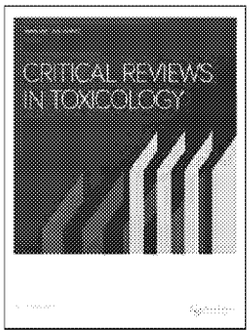

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REVIEW ARTICLE

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Does occupational exposure to formaldehyde cause hematotoxicity and leukemia-specific chromosome changes in cultured myeloid progenitor cells?

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ABSTRACT

Several cross-sectional studies of a single population of workers exposed to formaldehyde at one of two factories using or producing formaldehyde–melamine resins in China have concluded that formaldehyde exposure induces damage to hematopoietic cells that originate in the bone marrow. Moreover, the investigators interpret observed differences between groups as evidence that formaldehyde induces myeloid leukemias, although the mechanisms for inducing these diseases are not obvious and recently published scientific findings do not support causation. Our objective was to evaluate hematological parameters and aneuploidy in relation to quantitative exposure measures of formaldehyde. We obtained the study data for the original study (Zhang et al. 2010) and performed linear regression analyses. Results showed that differences in white blood cell, granulocyte, platelet, and red blood cell counts are not exposure dependent. Among formaldehyde-exposed workers, no association was observed between individual average formaldehyde exposure estimates and frequency of aneuploidy, suggested by the original study authors to be indicators of myeloid leukemia risk.

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Introduction

The International Agency for Research on Cancer (IARC) evaluated the carcinogenicity of formaldehyde in October, 2009, and according to Monograph 100F, *"on balance, the Working Group concluded that the epidemiologic evidence shows that occupational exposure to formaldehyde causes leukemia"* (IARC 2012). However, Monograph 100F noted that this determination was not unanimous, and a small majority viewed the evidence as sufficient of carcinogenicity while a minority viewed the evidence as limited. Monograph 100F further stated:

> Particularly relevant to the discussions regarding sufficient evidence was a recent study accepted for publication which, for the first time, reported aneuploidy in blood of exposed workers characteristic of myeloid leukaemia and myelodysplastic syndromes, with supporting information suggesting a decrease in the major circulating blood-cell types and in circulating haematological precursor cells. The authors and Working Group felt that this study needed to be replicated.

The specific study referred to here was "Occupational Exposure to Formaldehyde, Hematotoxicity, and Leukemia-Specific Chromosome Changes in Cultured Myeloid Progenitor Cells" by Dr. Luoping Zhang and 33 coauthors, accepted for publication one week before the IARC Working Group convened on 20 October 2009, and officially published online on 7 January 2010 (Zhang et al. 2010).

In 2010, the US Environmental Protection Agency (EPA) Integrated Risk Information System (IRIS) issued a Draft Toxicological Review of Formaldehyde (referred to hereafter as the IRIS Draft). Although the IRIS Draft has not yet been finalized, it stated that Zhang et al. (2010) provided *"the best evidence for bone marrow toxicity, where they report not only a*

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reduction in white blood cell counts, but reductions in cell counts of all the blood cells, as well as increased mean cell volume" (EPA 2010). The IRIS Draft also noted that

> ... the results of Zhang et al. (2010) need to be extended (analysis for additional chromosomal aberrations) and repeated. Although further evidence is needed to better understand the hypothesized mechanisms for formaldehyde-induced effects on hematopoietic stem cells, the observed hematologic effects in humans cannot be set aside. Therefore, however unlikely, the current data support the biological plausibility of formaldehyde effects on the hematopoietic system (EPA 2010).

This highly influential study – which has not been replicated to date – was a cross-sectional statistical evaluation comparing blood parameters considered by the authors to be indicators of hematotoxicity and chromosomal changes in myeloid progenitor cells, specifically monosomy 7 and trisomy 8. However, no actual changes in any of the parameters were measured; rather, differences between groups were observed. These parameters have been found to be more common among individuals with acute myeloid leukemia (AML) but are not associated with chronic myeloid leukemia (CML). Comparisons were made between 43 workers exposed to formaldehyde and 51 unexposed controls, based strictly on exposure represented as a dichotomous variable (exposed versus unexposed) and not considering individual level exposure data. The exposed group included workers if they had formaldehyde exposure levels "of about 1–2 ppm [parts per million] on most days during the initial screening" and had worked in the same job for the previous three months (Zhang et al. 2010). Of the 43 exposed subjects included in the study, 41 (95%) had worked for at least one year in either of two factories that produced or used formaldehyde-melamine resins. The formaldehyde exposure was characterized by the authors as "*relatively high levels of formaldehyde (mostly between 0.6 and 2.5 ppm)*" (Zhang et al. 2010). The 51 unexposed workers were selected from three separate workplaces (reported by Bassig et al. 2016 to be two clothes manufacturing factories and one food production factory) in the same region, with no occupational formaldehyde exposure (verified via workplace sampling), and frequency matched on age (± 5 years) and sex (Zhang et al. 2010).

Blood samples from these two groups of exposed and unexposed workers have been included in additional evaluations of aneuploidy and structural chromosome aberrations (SCAs) of all 24 chromosomes (Lan et al. 2015), and in comparisons of hematotoxicity, monosomy 7 in myeloid progenitor cells (MPCs) and B-cell activation biomarkers across groups exposed to benzene, formaldehyde, and trichloroethylene (Bassig et al. 2016). In all three publications, differences in blood parameters and genetic markers of the group of workers exposed to formaldehyde are compared with those of the unexposed group, i.e. ecologically. Specifically, in the report relied upon by the IARC Working Group (Zhang et al. 2010), statistically significant differences were reported for several blood parameters, as well as increased aneuploidies (monosomy 7 and trisomy 8) in myeloid progenitor cells in comparing results from formaldehyde exposed workers and unexposed controls (Zhang et al. 2010). The analyses were based on the OctoChrome FISH protocol developed and marketed by some of the same investigators (Zhang et al. 2005). Based on these findings, Zhang et al. (2010) proposed that formaldehyde exposure may have damaged hematopoietic cells, and therefore, provides support for the hypothesis that formaldehyde causes myeloid leukemia, and presumably AML specifically.

However, in none of these reports (i.e. Zhang et al. 2010; Lan et al. 2015; Bassig et al. 2016) are the individual formaldehyde exposure measures (or mean of these) among the "exposed" workers evaluated for their relationship, if any, with the reported outcome measures. Nor were the individual formaldehyde exposure estimates divided into ranges of exposure for analysis with the blood and aneuploidy outcomes as was done with benzene and trichloroethylene exposure estimates in the study by Bassig et al. (2016). Individual formaldehyde exposure measurements clearly were available, as each of the reports describes the sampling methods used, for example: "*Personal FA exposure was monitored with SKC UMEx 100 passive samplers, which were worn by workers in the exposed factories for a full work shift for about three workdays over a 3-week period*" (Bassig et al. 2016). Averages of the actual exposure measurements were used to estimate individual formaldehyde estimates for each of these workers. However, the authors ultimately treat all concentrations of formaldehyde exposure among the "exposed" workers as the same, despite a fourfold 10th–90th percentile exposure range (0.6–2.5 ppm), which the authors claimed was insufficient to differentiate risks by actual exposure levels: In a subsequent publication of the same underlying data, Lan et al. (2015) reported "*The study was designed to evaluate mechanistically relevant biomarkers in workers exposed to relatively high levels of FA, and as a consequence there was a relatively narrow range of exposure that precluded assessment of exposure-response relationships.*" However, the authors of these reports fail to consider that unmeasured differences between the exposed and unexposed groups – other than their formaldehyde exposure – likely contributed to the differences observed at the group level, and that some association, if present, would be seen across this more than fourfold range of individual exposure estimates and some of the blood and aneuploidy measures.

Gentry et al. (2013) obtained most of the Zhang et al. (2010) data from the National Cancer Institute (NCI), through a Freedom of Information Act (FOIA) request. The individual formaldehyde exposure measurement data, however, were not provided. In brief, the Gentry et al. (2013) re-analysis did not substantiate the original study claim that monosomy 7 and trisomy 8 arose *in vivo* in hematopoietic stem cells from humans exposed to formaldehyde. They noted that based on the kinetics of CFU-GM colony formation, the reported aneuploidies observed could not have arisen *in vivo*, but most likely occurred *in vitro* during cell culture (Gentry et al. 2013). This has been reiterated by Albertini and Kaden (2017). Gentry et al. (2013) also detected and reported significant methodological limitations, including the discovery that the authors did not follow their own protocol, which specified the number of cells to be scored from each study participant. This information was not included in the Zhang et al. (2010) publication and was only determined through the acquisition

of the raw data from the study through the FOIA request. In fact, cultures from only one and three exposed workers respectively met the criterion specified in the Zhang study protocol (Zhang et al. 2010) that a minimum of 150 cells would be scored for both the monosomy 7 and trisomy 8 evaluations (Gentry et al. 2013). Gentry et al. (2013) concluded that their reanalyses *"raise sufficient questions that limit the use of Zhang et al.* (2010) *to support the hypothesis that formaldehyde exposure is causally related to leukemia or lymphoid malignancies"* (Gentry et al. 2013). They also recommended that exposure–response analyses would be helpful in verifying that occupational exposure to formaldehyde *"damages hematopoietic stem or early progenitor cells in the bone marrow and/or peripheral blood"* as reported by Zhang et al. (2010) (Gentry et al. 2013).

In 2014, we requested the individual exposure measurement data for each of the participants in the Zhang et al. (2010) study. In 2016, our request was in part granted and the mean formaldehyde estimate for each exposed worker (but not the individual exposure measurement values) was provided via a Technology Transfer Agreement (TTA) with the NCI. In this report, we extend the Gentry et al. (2013) reanalysis using the additional data provided to perform exposure-response analysis.

Methods

Demographic and exposure characteristics of study subjects as reported by Zhang et al. (2010) were replicated. As reported by Zhang et al. (2010), formaldehyde exposure among the exposed subjects was estimated based on formaldehyde monitoring performed using diffusion samplers (limit of detection = 0.012 ppm) *"for a full shift (>240 min) on ~3 working days over a 3-week period."* For the exposed group, Zhang et al. (2010) reported the median of the summary 8-hour time-weighted average (8-h TWA) measurement and the 10th and 90th percentiles of the summary measurements. Using the summary TWA measurement for each exposed worker, we categorized participants into "lower" and "higher" exposure groups based on the overall median exposure level (1.3 ppm).

Zhang et al. (2010) reported that the assigned exposure values in controls were based on the 8-h TWA level in their respective control factories based on measurements performed for a subgroup of workers. Study subjects with non-detectable formaldehyde exposure were assigned a value of the limit of detection divided by the square root of two. Based on this information, seven non-exposed workers had been assigned 0.0085 ppm by Zhang et al. (2010), consistent with a limit of detection of 0.012 ppm. In addition, 14 unexposed workers had been assigned an intensity of 0.0146 ppm and 27 unexposed workers had been assigned an intensity of 0.0262 ppm as an 8-h TWA. Estimated exposures in the exposed workers were 8-h TWAs based on the arithmetic mean of the individual's exposure measurements (which were not provided by NCI) and ranged from 0.318 to 5.61 ppm among exposed workers (Figure 1).

As described (Zhang et al. 2010), peripheral blood samples were collected from study subjects in the workplace and from the formaldehyde exposed workers after they had been monitored at least twice. Complete blood counts with differential and lymphocyte subsets were measured for each study subject. Cells defined by the authors as hematological progenitor cells (peripheral blood mononuclear cells) were cultured using the colony forming unit-granulocyte/macrophage (CFU-GM) assay. Metaphases from CFU-GM cells were prepared after 14 days (d) of culture. Two types of chromosomal markers that the authors described as *"among the most frequent cytogenetic changes observed in myeloid leukemia and myelodysplastic syndromes,"* specifically, monosomy 7 and trisomy 8, were examined in CFU-GM cells using fluorescence *in situ* hybridization (FISH) staining of metaphase spreads (Zhang et al. 2005). Each metaphase spread was examined microscopically for 10 workers chosen from those with the highest formaldehyde exposure and 12 unexposed controls frequency matched to the exposed workers by age and sex (Zhang et al. 2010). Frequency matching allowed for the control of age and sex in the analysis.

In the present analysis, exposure values for each worker were linked with the eight blood count parameters

Table 1. Association between formaldehyde exposure and the blood parameters.

Blood parameter	Unadjusted Exp(β^a)	95% CI	p value	Adjusted Exp(β^a)[b]	95% CI	p^c value
WBC						
Unexposed	Reference			Reference		
<1.3 ppm	**0.85**[d]	0.76–0.96		**0.87**	0.78–0.97	
≥1.3 ppm	**0.86**	0.76–0.97	.992	**0.85**	0.76–0.96	.943
Lymphocytes						
Unexposed	Reference			Reference		
<1.3 ppm	**0.83**	0.73–0.95		**0.85**	0.75–0.96	
≥1.3 ppm	**0.80**	0.70–0.92	.890	**0.79**	0.69–0.90	.660
Monocytes						
Unexposed	Reference			Reference		
<1.3 ppm	0.86	0.72–1.04		0.90	0.77–1.06	
≥1.3 ppm	0.92	0.76–1.11	.856	0.89	0.75–1.04	.973
Granulocytes						
Unexposed	Reference			Reference		
<1.3 ppm	**0.86**	0.74–1.00		0.87	0.75–1.01	
≥1.3 ppm	0.89	0.76–1.04	.931	0.88	0.75–1.03	.997
RBC						
Unexposed	Reference			Reference		
<1.3 ppm	**0.94**	0.89–0.99		**0.94**	0.91–0.98	
≥1.3 ppm	**0.94**	0.89–1.00	.999	**0.94**	0.90–0.98	.947
Hemoglobin						
Unexposed	Reference			Reference		
<1.3 ppm	0.97	0.92–1.02		0.98	0.94–1.01	
≥1.3 ppm	1.00	0.94–1.05	.667	0.99	0.95–1.03	.818
Platelets						
Unexposed	Reference			Reference		
<1.3 ppm	**0.85**	0.76–0.96		**0.85**	0.75–0.96	
≥1.3 ppm	0.91	0.80–1.03	.695	0.91	0.80–1.03	.674
MCV						
Unexposed	Reference			Reference		
<1.3 ppm	1.03	0.99–1.07		1.03	0.99–1.08	
≥1.3 ppm	**1.06**	1.02–1.11	.379	**1.06**	1.02–1.11	.550

[a]Regression coefficient between log-transformed blood parameter and formaldehyde.
[b]Adjusted for combined sex/smoking variable.
[c]p values for pairwise comparison between <1.3 ppm and ≥1.3 ppm categories.
[d]Bolded results are statistically significantly different from the reference group.

Table 2. Monosomy of chromosome 7 (−7) and trisomy of chromosome 8 (+8) in peripheral blood cells scored by Zhang et al. (2010) – updated table from Gentry et al. (2013) and sorted by average intensity of formaldehyde.

FA ppm	Smoking status	Total cells scored	Abnormal metaphases −7	Frequency −7 (%)	Total cells scored	Abnormal metaphases +8	Frequency +8 (%)
Produced or used melamine formaldehyde resins ($n = 10$)							
5.61	No	109	4	3.7	139	0	0.0
2.68	Yes	76	9	11.8	149	1	0.7
2.60	Yes	123	20	16.3	173	4	2.3
2.32	No	39	6	15.4	61	2	3.3
2.29	Yes	274	11	4.0	180	4	2.2
2.00	Yes	132	15	11.4	192	2	1.0
1.99	No	50	10	20.0	78	2	2.6
1.94	No	95	3	3.2	108	0	0.0
1.38	No	101	4	4.0	53	0	0.0
1.38	No	61	13	1.38	33	0	0.0
Worked in control factories ($n = 12$)							
0.03	No	272	10	3.7	226	2	0.9
0.03	Yes	260	10	3.8	215	2	0.9
0.03	No	163	8	4.9	91	0	0.0
0.03	Yes	140	6	4.3	69	0	0.0
0.03	No	78	2	2.6	83	0	0.0
0.03	No	71	1	1.4	37	0	0.0
0.03	Yes	20	2	10.0	25	0	0.0
0.03	Yes	18	1	5.6	21	0	0.0
0.01	Yes	288	19	6.6	197	2	1.0
0.01	No	70	9	12.9	94	1	1.1
0.01	No	49	4	8.2	67	0	0.0
0.01	No	24	0	0.0	22	0	0.0

Shaded cells represent samples following reported methodology (analyzed ≥150 cells).

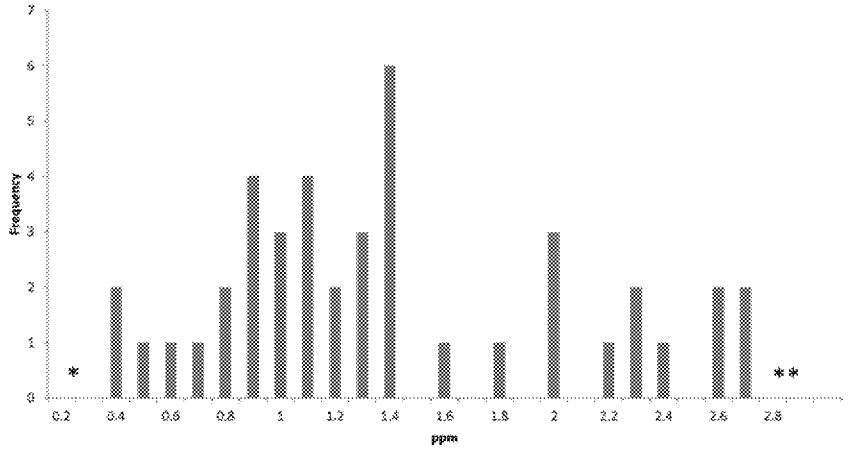

Figure 1. Distribution of inhalable formaldehyde measurements in workers ($n = 43$) from exposed factories. *Formaldehyde exposure for the 51 workers from unexposed workplaces is not included. **The maximum outlier of 5.6 ppm has not been included in this figure.

(discussed below), and, where applicable, the aneuploidy results. Characteristics of the study participants, including means, standard deviations, medians, and ranges for blood parameters were described and stratified by exposure status (exposed/not exposed to formaldehyde). This allowed further insight into variability of blood parameters among the study subjects (formaldehyde-exposed and controls) as well as identified potential confounders, when stratified by exposure status (exposed/not exposed to formaldehyde). In addition, median values for blood parameters and ranges were compared between exposed and unexposed groups, and individual values for blood parameters were compared to reference intervals for the Chinese population (Wu et al. 2015) to identify individual values that fall outside normal ranges. All analyses were conducted using SAS 9.3 (SAS Inc., Cary, NC).

Indicators of hematotoxicity

Each of the blood count parameters, specifically, white blood cell (WBC) count and its component lymphocytes, monocytes, and granulocytes; red blood cell (RBC) count and its component hemoglobin and platelets; and mean corpuscular volume (MCV), was examined as the primary outcome variables of interest. Results as reported in Zhang et al. (2010), i.e. comparing exposed and unexposed groups, were verified. Additional stratified analyses were conducted among the exposed group only using the median cut-point, as well as linear regression analyses using the individual exposure estimates and relevant covariates and the natural logarithm of the blood count data. Age, body mass index (BMI), sex, current smoking, current alcohol consumption, recent respiratory infections, recent use of Chinese medicine, and recent use of Western medicine all

were examined as possible covariates. Thalassemia trait was also considered and was defined as those blood samples with MCV values of less than 70 femtoliters (fl), as values below this level are believed to provide a possible indication of the thalassemia trait. As reported by Gentry et al. (2013), thalassemia, an inherited blood disease, decreases MCV and increases RBC counts, so thalassemia is a possible confounder of the association between formaldehyde exposure and RBC and MCV. To address this, we ran sensitivity analyses after excluding five workers with MCV levels of less than 70 fl.

The variables included in the adjusted models were guided by the descriptive analysis. The unexposed and exposed workers were similar in terms of age and sex, as would be expected as a result of the frequency matching that was used in selecting unexposed controls; however, only 14% of the study participants were women. Because there were no women who reported current smoking, we combined smoking and gender variables (into groups of male smokers, male non-smokers, and female non-smokers), allowing contrasts to be made by gender and smoking individually and jointly.

Aneuploidy

Zhang et al. (2010) reportedly analyzed monosomy 7 and trisomy 8 based on the percentage observed in each sample, which was determined by dividing the number of aneuploidies observed for each subject by the number of cells counted *in vitro*. The strong case challenging the biological rationale of the CFU-GM analysis presented by Gentry et al. (2013), as well as the very small sample sizes reported, argue against performing additional statistical analyses for these outcomes by individual formaldehyde exposure estimates. Nevertheless, we provide descriptive and graphical results in relation to test result, reliability (based on actual counts versus 150 required by the protocol), smoking and formaldehyde exposure estimate.

Results

Indicators of hematotoxicity

Among women classified as non-smokers, no differences in any of the blood parameters were detected between the exposed workers and unexposed workers (Supplemental Table 1). There were no women who smoked. Among men classified as non-smokers, WBC, lymphocyte, and RBC were higher in the unexposed workers compared with the exposed workers. Among male smokers, lymphocytes were higher and MCV was lower in the unexposed compared with the exposed. Mean blood parameters for exposed and unexposed workers were summarized according to gender and smoking status (Supplemental Table 1). As expected, smokers had higher WBC counts than non-smokers, irrespective of exposure status, although male non-smokers appeared to have higher WBC counts than female non-smokers for both exposed and unexposed workers. Among unexposed workers, for example, white blood cell counts were 5064.3 per μl in women (all non-smokers), compared with 6093.3 per μl in men who were non-smokers and 6796.5 per μl in men who were smokers. Statistically significant differences in means between exposed and unexposed workers were observed for WBC counts and RBC counts in male non-smokers, but not male smokers. Among both male smokers and male non-smokers, statistically significant differences in means between exposed workers and unexposed workers were seen for lymphocyte counts.

Although trend tests were statistically significant in untransformed models of WBC, RBC, and lymphocyte counts, exposure-dependent differences in these parameters were not apparent when formaldehyde exposure was categorized according to median concentration in the exposed workers, and adjusting for smoking and sex (Figure 2).

In log-transformed models of blood parameters adjusted for sex and current smoking, WBC, RBC, and lymphocyte counts were lower in the formaldehyde exposed workers compared with the unexposed workers, but the differences were of similar magnitude in both exposure categories (<1.3 ppm, ≥1.3 ppm) (Table 1). Specifically, compared with the unexposed, WBCs were 13–15% lower, lymphocyte counts were 15–21% lower, and RBCs were 6% lower. Additionally, MCV was 6% higher in the ≥1.3 ppm formaldehyde exposure category only compared with the unexposed, and platelet counts were 15% lower in the <1.3 ppm formaldehyde exposure category only compared with the unexposed. Results in unadjusted linear regression models were similar (Table 1).

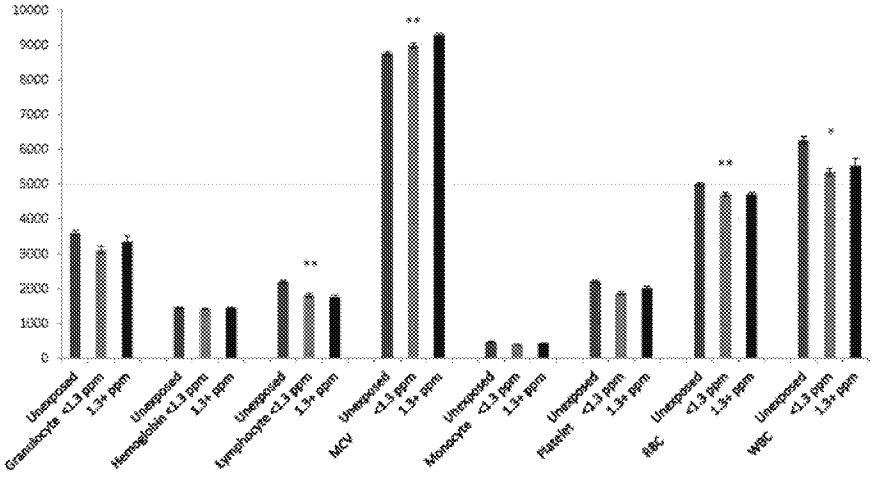

Figure 2. Cell counts per μl blood by formaldehyde exposure. Untransformed means/cells μl and standard errors. All models adjusted for sex and current smoking. Hemoglobin and MCV values are reported in g/dL and fl, respectively, and have been multiplied by 100 to make them visible. RBC values were multiplied by 1000. Platelets were multiplied by 100. *Ptrend <0.05; **Ptrend <0.01.

Sex and smoking were associated with some of the blood parameters in the adjusted models. Statistically significant differences in monocyte, RBC, and hemoglobin counts were detected for the combined variable of current smoking status and sex. Compared with male non-smokers, monocyte counts were 24% higher in male smokers ($p = .0002$) and 33% lower in female non-smokers ($p = .003$). RBC counts were 20% lower in female non-smokers compared with male non-smokers ($p < .0001$) and 4% lower in male smokers compared with male non-smokers ($p = .031$). Hemoglobin counts were 18% lower in female non-smokers compared with male non-smokers ($p < .0001$); no differences in hemoglobin were detected between male smokers and male non-smokers.

In the sensitivity analysis, removing five subjects with presumed thalassemia trait did not substantially modify the results for MCV or RBC. As noted previously, thalassemia, an inherited autosomal recessive blood disease common in Asian populations, is associated with a decrease in MCV and an increase in RBC counts. In addition, removing these individuals from consideration did not change the overall conclusions for any of the other blood parameters measured (data not shown).

We also generated models of blood parameters that included the exposed workers only with formaldehyde modeled as a continuous variable (Supplemental Table 2). No significant differences in any of the blood parameters were seen with each one ppm increase in formaldehyde exposure, adjusting for sex and smoking.

Finally, we compared means of the blood parameters for exposed and unexposed workers with the reference intervals for healthy Chinese adult men and women (Wu et al. 2015). We extended the comparison by Gentry et al. (2013) by identifying the number of workers in each category that fell outside of the reference interval (Supplemental Table 3). Although the sample size was small, which limited formal statistical analysis, few workers had blood count values that fell outside of the reference ranges. For WBC counts and its components (lymphocytes, monocytes, and granulocytes), none of the women fell outside of the normal ranges. Among men, two exposed workers had low WBC counts while one exposed worker and one unexposed worker had high WBC counts. No exposed men had lymphocyte counts that fell outside of the normal ranges. Four exposed men had monocyte counts that were higher than normal values; however, 13 unexposed men had monocyte counts that were higher than normal values. Three exposed men had granulocyte counts that were lower than normal values and one exposed man had granulocyte counts that were higher than normal values.

Aneuploidy

Zhang et al. (2010) analyzed monosomy 7 and trisomy 8 in a subset of 10 "highly exposed" workers and 12 matched controls (Table 2). These data are plotted according to average intensity of formaldehyde for monosomy 7 and trisomy 8 (Figure 3). Few subjects had adequate numbers of CFU-GM progenitor cells analyzed to meet the study protocol criteria of evaluating >150 cells. The lack of compliance with the study protocol is critical, as the cutoff or background for FISH results is expected to be above zero and no cutoff was established for this analysis. The normal cutoff for an analysis of 200 cells can be as high as approximately 5%, depending on the number of false positives identified in the normal specimens (Wolff et al. 2007). Typically, in the clinical setting 200–400 cells are scored and cutoffs determined based on the false positives previously defined from normal specimens.

When considering the protocol established by Zhang et al. (2010), for monosomy 7, only a single exposed worker and four controls met the criterion of scoring 150 cells, while for trisomy 8, only three exposed workers and three controls met the criterion (Table 2). In addition, considering that the cutoff for these analyses would not be zero and assuming it could potentially be in the range of 2–5%, approximately half of the monosomy 7 findings could be below the cutoff, with the majority of trisomy 8 findings below the cutoff. Regardless of the number of cells considered, however, no pattern between formaldehyde exposure and the frequency of monosomy 7 was observed (Figure 3). Sensitivity analyses revealed that the frequency of monosomy 7 in workers with fewer than 80 cells scored is highly sensitive to small changes in the number of cells included. For example, the frequency of monosomy 7 in a subject with 78 scorable cells would change by more than 1% with the detection of one additional (or one fewer) abnormality (e.g. actual: 2/78 (2.6%) to 3/78 (3.8%) with one additional abnormality detected) and the uncertainty proportionately greater with fewer counts. This highlights the potential impact of results from subjects for which the appropriate number of cells (based on the criterion defined by Zhang et al. 2010) were not scored.

No pattern between formaldehyde exposure and trisomy 8 was observed (Figure 3). Of note, all the selected exposed workers who additionally met the research protocol were also smokers.

Discussion

The Zhang et al. (2010) study was highly influential in the evaluation of formaldehyde as a plausible human leukemogen, and as noted above, was specifically cited by IARC (2012) and in the draft EPA (2010) formaldehyde IRIS assessment as providing evidence to support plausible mechanisms by which formaldehyde exposure may cause leukemogenesis. This recognition came despite the fact that primary evaluations reported by Zhang et al. (2010) of aneuploidies and indicators of hematotoxicity were limited to fairly crude aggregation of workers from different industries into "exposed" and "unexposed" categories. However, the most serious problems underlying the study may not have been apparent to the evaluation committees, because the limitations regarding analyses of dichotomous formaldehyde exposure (exposed versus unexposed), as well as measurement of aneuploidy (whether the reported aneuploidies could have occurred during cell culture *in vitro)* were not reported by the original authors. The study investigators also failed to acknowledge that the differences seen between the exposed and unexposed groups could reflect other underlying differences between the employees at different study factories. Additional information about the two groups beyond the few available occupational

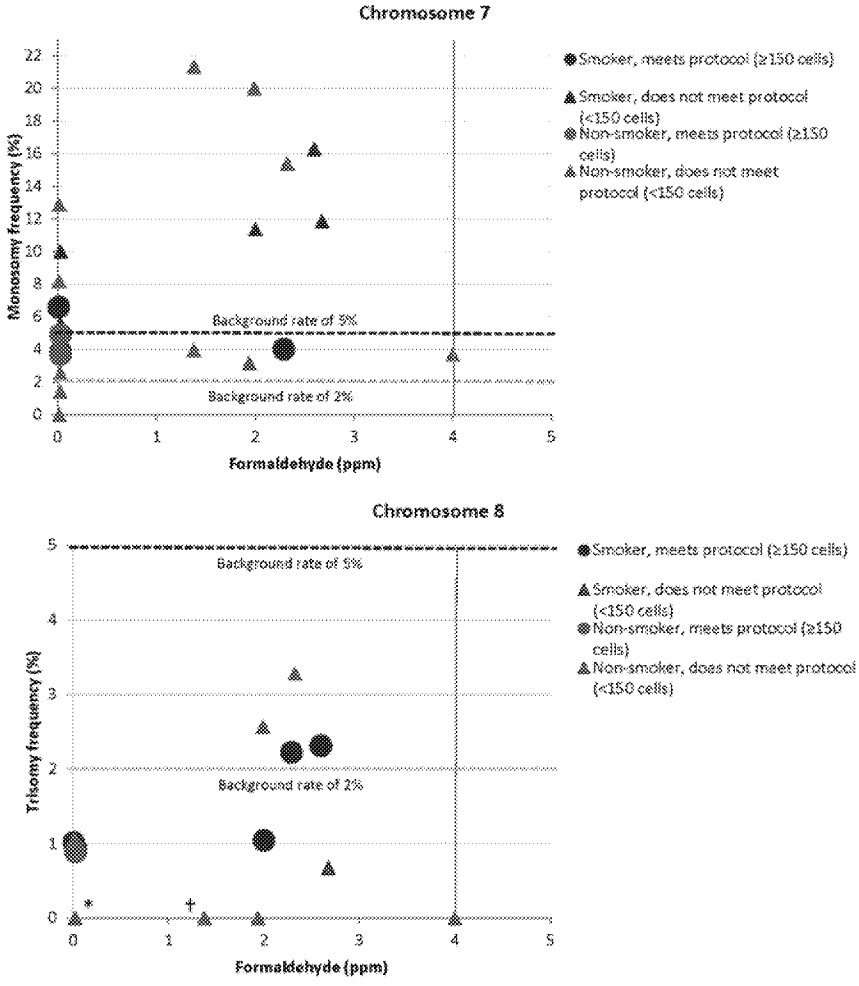

Figure 3. Individual average formaldehyde intensity for monosomy 7 and trisomy 8. * Represents 8 subjects. † Represents 2 subjects. Vertical line represents the maximum value of 5.6 ppm, plotted at 4 ppm to improve readability of the figure.

and individual characteristics would be needed in order to more fully evaluate these differences. Furthermore, the publication was not generally available for full review and evaluation prior to the IARC working group meeting. Zhang et al. (2010) was accepted for publication one week before the meeting, and published online two months after the meeting (Baan et al. 2009). Study data were not shared for re-evaluation until years after they were requested (Gentry et al. 2013). However, the EPA (2010) IRIS assessment for formaldehyde is still in draft form, and the results of the analyses presented here should help place the original interpretations of the reported study findings into the proper context.

One of the major criticisms of the Zhang et al. (2010) study noted by Gentry et al. (2013) was the decision not to present any results by estimated individual exposure level, which would have provided a fuller evaluation and much stronger evidence of an association, should one exist. Although Gentry et al. (2013) had obtained most of the Zhang et al. (2010) study data through a FOIA request, the raw exposure measurement data and the summary variable were withheld. This prevented fuller evaluation using individual exposure estimates. The individual mean exposure estimates, however, eventually were provided to us by NCI, and we have completed and reported here the results of a fuller evaluation. One limitation in our re-analysis (as well as the initial analysis) reflects the underlying assumptions associated with a single summary measure of average formaldehyde exposure based on one to three samples collected during the three week period prior to biological sampling for each study subject. It is unknown whether these exposure measurements reflect long-term exposure levels; however, the expected timeframe for exposures to impact the reported blood parameters may be fairly recent.

Indicators of hematotoxicity

Zhang et al. (2010) reported lower WBC, lymphocyte, granulocyte, platelet, RBC, and monocyte counts in the exposed workers compared to the unexposed workers. If these differences in fact were due to formaldehyde exposure, we would expect to see exposure-dependent differences in these blood parameters across the nearly seven-fold range (0.4–2.7 ppm, excluding the highest value 5.6 ppm) of mean measured individual exposures among exposed workers. Leukemogenic effects, as seen with benzene and alkylating agents, may not correlate closely with exposure, but rather reflect individual genetic predispositions. However, these factors would be expected to be equally distributed between valid comparison, i.e. exposed and unexposed, groups. Although blood parameter values were lower for workers in the formaldehyde exposed group compared with the control workers overall,

differences for granulocyte, platelet, and WBC counts were greater for the workers exposed to formaldehyde concentrations <1.3 ppm than for workers exposed to formaldehyde concentrations ≥1.3 ppm (Figure 2). Given that we would also expect to observe consistent declines in these relationships across levels of exposure intensity among the exposed workers, we performed regression analyses among the exposed workers only (Supplemental Table 2). There was a clear and consistent lack of any association with formaldehyde. That sex and/or smoking were associated with the blood parameters – and in some cases statistically significantly so – suggests that true associations with formaldehyde, if present, would be suggested as well.

Differences in blood parameters are not themselves indicators of leukemia risk. Unusually high or low blood parameter values typically are signs and symptoms of other conditions or diseases. In conjunction with other diagnostic tests, blood count data are used in the clinical evaluation of existing leukemia (American Cancer Society 2016). However, the range of values for exposed and unexposed workers were similar, and no obvious effect of formaldehyde exposure can be seen (Supplemental Table 3). In addition, the mean and maximum values for monocytes (in particular) were higher in unexposed men than exposed men; however, any clinical significance of these results is unlikely and conclusions cannot be drawn from such a small sample size. It would be remarkable if modest differences in these parameters seen in cross-sectional samples of any population were actually predictive of leukemia risk.

Measurements of eosinophils and basophils, components of white blood cells, were not available in the data provided. Although counts of lymphocytes and monocytes were measured, it does not appear that granulocytes were identified and counted by type: eosinophils, basophils, and neutrophils.

Other factors can influence WBC counts, including infections, immune system disorders, and smoking. Smokers consistently have higher WBC counts than non-smokers and the WBC counts increase with smoking level (Sunyer et al. 1996). Smokers may have elevated hemoglobin consequent to an increase in carboxyhemoglobin levels and some increase in neutrophils, but this change in hemoglobin does not explain smokers' increased risk of AML or MDS. Higher WBC counts are also associated with coronary heart disease deaths, independent of the effects of smoking on heart disease (Brown et al. 2001). Dietary factors that influence blood parameters were also unmeasured, for example vitamin B12 or folate deficiency, which are associated with low WBC counts. Future studies should address the limitations of the Zhang et al. (2010) study, including small sample size; poorly controlled comparator populations (since other factors such as workplace stress or differences in genetic predisposing factors could contribute to the subtle differences reported between groups); and temporally-remote toxic endpoints, e.g. a few months to several years for leukemia to develop following exposure to benzene or oncolytic agents.

Aneuploidy

The identification of several serious methodological problems with the original study (Speit et al. 2010; Kuehner et al. 2012; Gentry et al. 2013; Albertini & Kaden 2017) already cast serious doubt on the validity of the findings, specifically with respect to monosomy 7 and trisomy 8, which are genetic anomalies claimed by Zhang et al. (2010) to indicate the biological plausibility that formaldehyde causes leukemia, and presumably AML specifically, as these aneuploidies may not be associated with other myeloid leukemias. As noted above, and as outlined by Zhang et al. (2010), there is considerable uncertainty in drawing conclusions based on analysis of aneuploidies. These are compounded given the methodological issues and resulting loss of study participant data due to failing to meet (or come close to) the counting criteria required by the study protocol. Consistent with methods recently advocated for visualizing data and elucidating bias (Lash et al. 2014) – in this case, over-interpretation bias – we chose to graphically plot the individual aneuploidy results (Figure 3), indicating for which individuals the counting rules were met, by individual formaldehyde exposure estimate. These graphs reinforce the broader conclusion that no confident interpretation of these findings can be made with respect to the possible role, if any, of formaldehyde in causing these aneuploidies. By extension, this illustrates the importance of transparency in study methods, quality control measures, and skepticism in causally interpreting ecological correlations. A full evaluation of the available data provides no basis for concluding that formaldehyde exposure causes leukemia and AML specifically.

The study participants and their data originally used by Zhang et al. (2010) to evaluate correlations between groups of formaldehyde-exposed and unexposed workers and several blood parameters and aneuploidies also have been included in further studies published by Lan et al. (2015) and Bassig et al. (2016). The blood samples used in these evaluations were collected prior to 2009. Lan et al. (2015) expanded the genetic analysis to evaluate frequency of monosomy, trisomy, and tetrasomy, as well as structural changes, for all 24 chromosomes. They also increased the number of subjects for which CFU-GM progenitor cells were cultured from blood samples collected in 2006 and stored for many years (Albertini & Kaden 2017), resulting in a total of 29 formaldehyde-exposed and 24 unexposed workers. The investigators used the same OctoChrome FISH protocol (Zhang et al. 2005) and again reported that at least 150 metaphases per slide were scored for subjects included in this report, as was erroneously stated in their earlier report (Zhang et al. 2010); however, we do not have access to these additional data to verify that the counting rules required by the protocol were followed. Although the analysis by Lan et al. (2015) offered an opportunity to address the critiques of others (Speit et al. 2010; Gentry et al. 2013), the authors offered insufficient details regarding their actual methods to verify any improvements. For example, it is unknown if the results from the 10 exposed workers and 12 controls were re-used or if new cells were cultured. These raise serious doubt regarding the validity of the reported findings (Lan et al. 2015). Nevertheless, the authors interpret their findings as *"further evidence that leukemia-related cytogenetic changes can occur in the circulating myeloid progenitor cells of healthy workers exposed to FA, which may be a*

potential mechanism underlying FA-induced leukemogenesis" (Lan et al. 2015). However, due to the potentially overlapping and, therefore, non-independent study sample, the results from this study cannot be relied upon to replicate or validate the results from the Zhang et al. (2010) study, and potentially propagate the ecological bias.

Lan et al. (2015) acknowledged two limitations. First, they noted the possibility that chromosomal abnormalities detected in CFU-GM may have arisen during the 14-d cell *in vitro* culture period, rather than being formed in the bone marrow *in vivo* and present in the circulating myeloid progenitor cells in the study subject. This criticism has been noted by others (Speit et al. 2010; Gentry et al. 2013; Albertini & Kaden 2017). The authors address this criticism by stating that if the abnormalities arose during the 14 d cell *in vitro* culture period, then workers exposed to formaldehyde would still exhibit a "greater tendency" to develop abnormalities during cell growth compared with control workers unexposed to formaldehyde, i.e. there is still a significant association with formaldehyde, and such events also "*support the leukemogenic potential of FA.*" However, no analyses to establish a relationship between the reported effects and individual formaldehyde exposure were presented.

Second, Lan et al. (2015) stated that formaldehyde exposure–response analyses were not conducted due to a narrow range of the intensity of formaldehyde. They noted that "*further studies of populations exposed to a wider range of FA concentrations are needed to address dose–response* in vivo." We note, however, that the range of formaldehyde exposures reported for the workers was relatively large and to relatively high average intensities, to which human populations are rarely exposed today in the US or Europe, even in occupational settings. The US Occupational Safety and Health Association (OSHA) permissible exposure limit (PEL) is 0.75 ppm (8-h TWA) and the American Council of Government and Industrial Hygienists (ACGIH) has adopted a threshold limit value ceiling (TLV-C) limit of 0.3 ppm. In fact, Lan et al. (2015) reported more than a three-fold difference in values between the 90th and 10th percentiles of the 29 exposed workers (2.61 ppm and 0.78 ppm, respectively), while Zhang et al. (2010) reported a similar four-fold difference in values between the 90th and 10th percentiles of 43 exposed workers (2.51 ppm and 0.63 ppm for the 90th and 10th percentiles of exposed workers, respectively). Similar median exposures were reported as well: 1.38 ppm for 29 exposed workers in Lan et al. (2015) and 1.28 ppm for 43 exposed workers in Zhang et al. (2010). Although these ranges may be adequate to evaluate exposure–response associations, the small sample size still may limit the ability to detect any true exposure–response relationships.

Lan et al. (2015) reported confirming the earlier finding of formaldehyde-associated monosomy 7, and also reported an increased frequency of trisomy 8 that was not statistically significant; however, the study population was not independent and the same blood samples were used to culture CFU-GM metaphases. Again, replication, or confirmation would require similar analyses conducted in other individuals or populations exposed to formaldehyde that are not already part of the study.

Conclusions

The IARC has reported that mechanistic data can be pivotal when the human data are not conclusive for carcinogenicity. This certainly remains true, although the epidemiology addressing occupational formaldehyde exposure and acute myeloid leukemia risk has improved since the IARC Working Group review of the evidence for formaldehyde carcinogenicity in 2009 (IARC 2012). Evidence available since the IARC review includes updated studies of the British chemical workers cohort: "*Our results provide no support for an increased hazard of myeloid leukemia…*" (Coggon et al. 2014) and US garment workers: "*We continue to see limited evidence of an association between formaldehyde and leukemia. However, the extended follow-up* [of the US garment workers] *did not strengthen previously observed associations*" (Meyers et al. 2013). From the largest study to date of over 15,000 incident acute myeloid leukemia cases and exposure to occupational exposure to solvents, no association was seen with formaldehyde exposure after adjusting for solvent exposure and ionizing radiation (Talibov et al. 2014). Furthermore, a re-analysis of US industrial workers exposed to formaldehyde (Beane Freeman et al. 2009) concluded, "*Findings from this re-analysis do not support the hypothesis that formaldehyde is a cause of AML*" (Checkoway et al. 2015). Taken as a whole, the epidemiological evidence from the most recent analyses and follow-up of available cohorts provides little if any evidence of a causal association between formaldehyde exposure and AML.

As the animal toxicological data are negative, a third line of evidence – mechanistic data – remains to be considered. The main cluster of studies published to date that evaluate hypothesized mechanisms are primarily based on the same biological samples analyzed and reported here (Zhang et al. 2010; Hosgood et al. 2013; Lan et al. 2015; Bassig et al. 2016). The additional evaluation of the underlying data including individual average measurements of formaldehyde exposure, however, demonstrates no association between level of formaldehyde exposure among the "exposed" workers and any of the blood parameters. This further challenges the utility of the Zhang et al. (2010) study and its progeny for elucidating potential formaldehyde leukemogenicity. All of the modes or mechanisms of action that have been proposed involve an impact on circulating blood cells, and not on the bone marrow, and how differences observed between groups might lead to AML has not been determined.

A direct genotoxic effect on the bone marrow, resulting in an impact on circulating cells, has been all but disproved (Lu et al. 2011; Moeller et al. 2011; Yu et al. 2015; Lai et al. 2016) based on the inability of exogenous formaldehyde to move beyond the portal of entry. The remaining hypothesized mechanisms of action involve an impact on circulating stem cells at the portal of entry. While Zhang et al. (2010) proposed that formaldehyde exposure leads to aneuploidy, the results from the current analyses indicate that exogenous formaldehyde exposure is not associated with the aneuploidies examined. Therefore, while Zhang et al. (2010) has been cited heavily to support the biological plausibility of formaldehyde as a cause of human leukemia, fuller analysis of the original study data verifies methodological limitations with respect to

monosomy 7 and trisomy 8, while demonstrating no association between individual exposure levels and several blood parameters among those occupationally exposed to formaldehyde. Moreover, a true aneugenic effect would also be seen at high concentrations used *in vitro*, and independently of the cell line used. Speit et al. (2010) attempted to replicate the *in vitro* effects reported by Zhang et al. (2010) using a different cell line and reported formaldehyde did not induce aneuploidy, while two positive controls (colcemid and vincristine) did induce aneuploidy. Separately, Kuehner et al. (2012) reported that colony forming ability was not reduced in myeloid progenitor cells in the presence of formaldehyde. Kuehner et al. (2013) reported that the gene expression profile of formaldehyde does not resemble that of known aneugens and more closely resembles that of known clastogens. Therefore, IARC's interpretation of the Zhang et al. (2010) study and the implications on the formaldehyde hazard classification should be reconsidered in light of the fuller evaluation of all of these data, and the updated EPA IRIS report should reflect the limited inferential value of the Zhang et al. (2010) study or any of its progeny (Hosgood et al. 2013; Lan et al. 2015; Bassig et al. 2016) until the scientific validity of each can be demonstrated. In particular, unmeasured factors – including workplace factors – that are distributed differently between the exposed and unexposed workers may explain differences noted in blood parameters and aneuploidies in the original results. IARC (2012) called for the replication of the Zhang et al. (2010) study. We suggest it be replicated using a new study population, actual measured formaldehyde exposures, and valid laboratory tests not subject to methodological problems such as deviation from protocol standards or complicated by questions of the origin (i.e. *in vivo* versus *in vitro*) of the effects.

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Declaration of interest

Authors: The design, analyses, presentation of results, interpretations and conclusions reflected in this paper are solely those of the authors. The employment affiliations of each of the authors is as shown on the cover page. We believe that there are no conflicts of interest for any of the authors to disclose related to the work performed in preparing and submitting this manuscript. Most of the authors (Mundt, Gallagher, Dell and Gentry) are full-time employees of Ramboll Environ US Corporation, and conducted work related to this paper as part of their normal employment. Ramboll Environ US Corporation is a consulting firm providing services in environmental and health sciences matters to private firms, trade organizations, and government agencies. Dr. Boffetta (Professor of Medicine, Hematology and Medical Oncology; Professor of Oncological Sciences and Professor of Environmental Medicine & Public Health at the Icahn School of Medicine at Mount Sinai) provided advice on the approach and design of the statistical analysis of the data, the interpretation of the results, and the preparation of the manuscript as an independent consultant to Ramboll Environ with fee for service. Dr. Natelson (Hematologist/Oncologist at Houston Methodist Hospital) provided assistance interpreting the hematological data and technical review on the manuscript and its content as an independent consultant, *pro bono*.

The authors had sole responsibility for the analyses performed, the interpretations made, conclusions drawn and the writing of the paper, which may not necessarily reflect the views of the sponsor. None of the authors has appeared as expert in any formaldehyde litigation or involved with or appeared in regulatory proceedings related to the contents of this paper. It is anticipated, however, that regulatory authorities will consider the contents of this study in making regulatory decisions relevant to the carcinogenicity of formaldehyde.

Presentation of findings: The analyses presented in this paper were based on data provided by the National Cancer Institute, as acknowledged above, and results have not been previously published. However, some of the findings reported here were presented to EPA IRIS staff on November 7, 2016 and the slides related to this publication (specifically slides 14–16) are posted on the EPA website, IRIS Calendar, Meetings Requested by Specific Members of the Public (https://cfpub.epa.gov/ncea/iris2/events.cfm).

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Supplemental material

Supplemental data for this article can be accessed here.

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